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Extragonadal Effects of Follicle-Stimulating Hormone on Osteoporosis and Cardiovascular Disease in Women during Menopausal Transition

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Trends in Endocrinology and Metabolism

Extragonadal-effects of FSH on osteoporosis and cardiovascular disease in women

--Manuscript Draft--

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Abstract:	<p>The risk of osteoporosis and cardiovascular disease increases significantly in postmenopausal women. Until recently, the underlying mechanisms have been primarily attributed to estrogen decline following the menopause. However, FSH levels rise sharply during menopausal transition and are maintained at elevated levels for many years. FSHR has been detected in various extragonadal sites, including osteoclasts and endothelial cells. Recent advances show that FSH may also contribute to postmenopausal osteoporosis and cardiovascular disease. We review here the key actions through which FSH could contribute to osteoporosis and cardiovascular disease in women transitioning through the menopause. Advancing our understanding of the precise mechanisms through which FSH promotes osteoporosis and cardiovascular disease may provide new opportunities for improving quality of life for postmenopausal women.</p>



School of Basic Sciences, Guangzhou Medical University
Laboratory of Molecular Endocrinology

Guangzhou, 03.06.2018

**To Editor-in-Chief,
Trends in Endocrinology and Metabolism**

Dear, Dr Beymer

We are extremely grateful for your prompt dealing with our manuscript entitled 'Extragonadal effects of FSH on osteoporosis and cardiovascular disease in women'. We thank the reviewer and the editor very much for their constructive comments on our manuscript. In light of these comments we have made amendments and prepared a revised manuscript. This has entailed editing and adding new text and figures, with changes highlighted in red. We have listed each of the reviewer's and the editor's comments and provided our detailed response.

We believe our manuscript is much improved and we hope that it is now considered suitable for publication in TEM.

Yours sincerely,

On behalf of the authors,

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Authors' response to the editor and the reviewer's comments: TEM-D-18-00111

EDITORIAL COMMENTS:

OVERALL ASSESSMENT

Very interesting insights on the role of FSH in osteoporosis and cardiovascular disease risk. Will appropriate revisions it will be a good resource for the broad readership of TEM.

Authors' response:

We thank the editor for such positive comments on our manuscript.

LENGTH

Currently at a length of ~2100 words, being on the shorter side of TEM reviews. Thus in addressing the reviewer comments, the main text can be expanded to between 3500 and 4000 words (final) if needed.

Authors' response:

We have revised and expanded our manuscript according to the editor's and the reviewer's comments. The length of our revised manuscript is ~2800 words.

CLARITY/ACCESSIBILITY

Please review the changes I have suggested in the main text and make any modifications necessary if the intended scientific meaning has been altered.

Authors' response:

We thank the editor's comments on our main text and we have made amendments accordingly.

Editor comment 1:

It is well know that Estrogen is a negative regulator of FSH secretion, is there evidence that the menopausal decline in E2 is not the main causative factor of elevated FSH levels in menopause?

Authors' response: We thank the editor for this comment. In addition to Estrogen, the glycoprotein hormone inhibin A and inhibin B have also been shown to suppress the production of FSH in a manner of negative feedback [ref 20, 21 in the main text]. Indeed, previous studies have shown that both decreased levels of estrogen and inhibins contribute to the elevated levels of FSH during the menopause transition [1]. We have included additional text in our revised manuscript as below (page 19, box 1, line 11-12).

“In addition, previous studies have shown that inhibin A and B also inhibits the production of FSH in a manner of negative feedback [20, 21].”

Editor comment 2:

This is only needed after the first glossary word.

Authors' response: We have removed them in our revised manuscript, except the first glossary word.

Editor comment 3:

Is there any information on the dynamics of the changes does FSH rise before the drop in estrogen levels?

Authors' response: We thank the editor for this comment. Previous studies have shown that FSH rises before the drop in estrogen levels during menopausal transition [ref.2 in the main text]. We have included a new introduction section in our revised manuscript (page 3-4, line 61-96). In the introduction, we have provided detailed information about hormonal changes in women with menopausal transition and statistics regarding incidence of rates of osteoporosis and CVD in pre- vs post-menopausal women. We have also provided figure 1 for demonstrating FSH rises before estrogen.

“Introduction

During menopausal transition, women undergo irregular menstruation or infrequent menses, with the permanent cessation of menstrual cycle due to depletion of viable follicles [1]. The length of menopausal transition is highly variable, ranging from 3-9 years in women aged around 45–55 years old. Menopausal transition is associated with alterations in hormone profiles. The two well established hormonal profiles are (1) a marked decline in estradiol and (2) a notable rise in follicle-stimulating hormone (FSH). The Study of Women's Health Across the Nation (SWAN), the most comprehensive U.S. study to date, followed premenopausal women over the menopause transition. Previous studies examined 1,215 SWAN participants who transitioned through the menopause over time and reported that FSH levels increased as early as 6 years before the final menstrual period (FMP) and stabilized 2 years after the FMP. Estrogen levels declined until 2 years before the FMP and stabilized after 2 years around the FMP [2] (Figure 1). Across the menopausal transition, serum levels of FSH increased from 15.15 mIU/mL to 98.21 mIU/mL, while serum levels of estradiol dropped from 60.26 pg/ml to 19.12 pg/ml [2].

Osteoporosis is defined as a skeletal disease with a significant reduction in bone mineral density and an associated increased risk of bone fracture [3]. Menopause is widely recognized as a risk factor for osteoporosis and bone fracture in women's later life. It has been reported that bone loss accelerates during the late stage of menopausal transition and continues into old age [4-6]. A large cohort of UK women study has shown that the relative risk (RR) of hip fracture in postmenopausal women is significantly higher than in premenopausal women with similar age (adjusted RR 2.22, 95% confidence interval [CI] 1.22–4.04; $p=0.009$) [7]. Menopause is also thought to be a risk factor for cardiovascular disease in women. The Framingham Heart Study reported that postmenopausal women aged 50–59 years had 4 times the 10-year incidence of coronary heart disease as premenopausal women of similar age, but results were not adjusted for age or smoking [8]. The Nurses' Health Study reported that women who had undergone bilateral oophorectomy showed an increased risk of coronary heart disease compared to premenopausal women (rate ratio, 2.2; 95 percent confidence limits, 1.2 and 4.2) [9]. Interestingly, recent studies have reported elevated risk factors for cardiovascular disease including body fat composition [10], lipids and lipoprotein [11], and vascular remodeling [12-13] in women transitioning through the menopause. Increased

susceptibility to osteoporosis and cardiovascular disease in postmenopausal (see Glossary) women may be associated with changes in their hormone levels.”

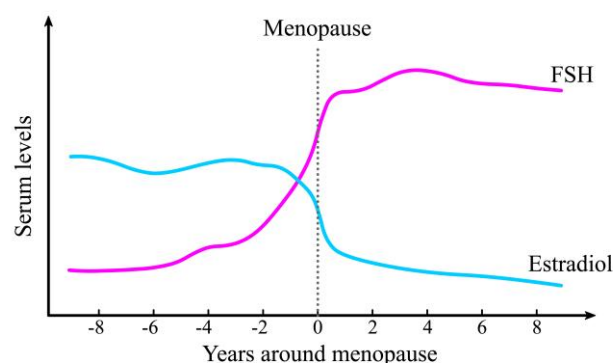


Figure 1. Ovarian hormonal changes in perimenopause. With transition into menopause, FSH increases markedly and estrogen declines. In postmenopausal women, estrogen levels are low, and FSH levels remain high for several years [2]. This graph is intended to show the trends of changes in FSH and Estrogen levels and do not stand for absolute amounts.

Editor comment 4:

We encourage you to contribute any of these optional features along with your revised manuscript:

Figure360 video: Create a narrated, animated version of one of your figures that helps the reader zoom in on the most important take-home message in two minutes or less. The video should contain data and panels from only one figure, and include minimal introduction. For further guidelines and examples, please click [here](#).

Interactive Questions (IQ): Use our online “Interactive Questions” tool to create a brief multiple-choice quiz, based on your review, that will be presented to readers alongside the published article as an interactive quiz to test their knowledge.

Authors’ response: A Figure360 video for Figure 4 is provided in our revised manuscript, which demonstrates the potential mechanisms through which FSH increases the risk of cardiovascular disease in women.

REVIEWER COMMENTS:

Reviewer #1:

I strongly support the publication

Authors’ response: We thank the reviewer for such positive comments on our manuscript.

Reviewer #2:

The present manuscript article by Zhu et al. presented a brief overview of the extragonadal actions of FSH specifically related to its actions in bone and the cardiovascular system. This review is timely given the recent publications in mice models indicating that the rise in FSH, rather than the decline in estradiol, may contribute to adiposity and loss of bone. Overall, the article succinctly presents most of the leading evidence in this newly emerging field. The

article is mostly well written, although some re-organization and editing for clarity and English are warranted as detailed below. One major limitation to the article in its current form is the tone that FSH undoubtedly plays a primary role in the development of osteoporosis and cardiovascular disease, when in fact, most research in this area has been conducted in animal or cell models, with little prospective evidence of translation across species, including humans. In fact, a recent review article regarding the extragonadal actions of FSH authored by T. Raj Kumar, a leading expert in the field concludes, "Human studies are currently controversial, and definitive answers are yet to come. If the extragonadal action of FSH is also confirmed in humans, novel therapeutic opportunities for treating postmenopausal osteoporosis and increased adiposity will be feasible." (Kumar TR. Endocrinology, 2018, 159(1):2-8). Thus, the state of the field remains somewhat unsettled. Much of this uncertainty is resultant to the inability of independent research groups to reproduce some of the studies reporting the detection of the FSH receptor (FSHR) in extragonadal tissues. Thus, the extragonadal distribution of FSHR, and if all extragonadal actions of FSH occur exclusively through FSHR, remain uncertain, especially in humans. Our comments below are broken into General (comments that apply to many sections of the paper and will not necessarily be pointed out at each individual instance) and detailed comments.

General Comments:

- 1) Throughout the manuscript, the FSHR is referred to as the sole receptor for FSH. This is not necessarily known as an absolute truth and should be tempered throughout the paper.
- 2) Manuscript could be structured better so that it is easy to follow.
- 3) Authors need to be careful referring to data in rodents as postmenopausal osteoporosis or CVD because rodents don't go through menopause. The model used (e.g., ovariectomy) should be stated and described as a model of menopause.

Authors' response:

We thank the reviewer for their comments. In our revised manuscript, we have made amendments accordingly. These includes:

- (1) We have referred to FSHR as the receptor of FSH.
- (2) We have re-structured our manuscript accordingly.
- (3) We have revised our manuscript accordingly and the rodent models are stated and described across our manuscript where necessary.

Detailed Comments:

1) Highlights -

- a. #5 - FSH represents a [potential] novel therapeutic target for the treatment of postmenopausal osteoporosis and CVD. 'Potential' should be added to this statement, because more research is needed before it is certain of its potential utility in humans.

Authors' response: We have added 'potential' in our revised manuscript (Highlights, line 11-12).

“FSH represents a potential novel therapeutic target for the treatment of postmenopausal osteoporosis and cardiovascular disease.”

2) Abstract -

a. Line 4 - Editing for English: "However, follicle stimulation hormone[s] levels rise sharply during the menopausal and [are maintained] at elevated levels for many years.

Authors' response: We have amended this sentence in our revised manuscript (page 2, line 35-36).

“However, follicle-stimulating hormone (FSH) levels rise sharply during menopausal transition and are maintained at elevated levels for many years.”

b. Line 5 - Remove the phrase “sole receptor for FSH”

Authors' response: We have removed the phrase ‘sole receptor for FSH’ through our revised manuscript.

c. Line 9 - "We review here the key actions of FSH in postmenopausal women and discuss underlying mechanisms". This review is really not specific to postmenopausal women per se, thus, suggest that this be tempered and say either just women, or "We review here the key actions by which FSH could contribute to the development of osteoporosis and CVD in women as they transition through menopause. - This same comment can be made about the title

Authors' response: We have amended our text in the abstract as below (page 2, line 38-40).

“We review here the key actions by which FSH could contribute to the development of osteoporosis and cardiovascular disease in women as they transition through menopause.”

We have amended our title as below (Page 1, line 1).

“Extragenital effects of follicle-stimulating hormone on osteoporosis and cardiovascular disease in women with menopausal transition.”

d. Line 11 - "disease may provide new opportunities for improving [quality of life] for postmenopausal women.

Authors' response: We have corrected it as below (page 2, line 43).

“....disease may provide new opportunities for improving quality of life for postmenopausal women.”

3) Introduction -

a. An introduction is needed to set up the manuscript. The intro could contain details on how sex hormones change across the menopausal transition including a figure that demonstrates how FSH rises before estradiol declines during early perimenopause and how FSH stays high and E2 low postmenopause.

Authors' response: We agree with the reviewer's comments. We have included an introduction section and a figure in our revised manuscript (page 3-4, line 61-96).

b. Inclusion of statistics regarding incidence of rates of osteoporosis and CVD in pre- vs post-menopausal women could be used here to highlight the importance of the problem.

Authors' response: We thank the reviewer for their suggestion. We have now included statistics regarding incidence of rates of osteoporosis and CVD in pre- vs post-menopausal women in our revised manuscript (page 3-4, line 82-96).

“A large cohort of UK women study has shown that the relative risk (RR) of hip fracture in postmenopausal women is significantly higher than in premenopausal women with similar age (adjusted RR 2.22, 95% confidence interval [CI] 1.22–4.04; $p=0.009$) [7]. Menopause is also thought to be a risk factor for cardiovascular disease in women. The Framingham Heart Study reported that postmenopausal women aged 50–59 years had 4 times the 10-year incidence of coronary heart disease as premenopausal women of similar age, but results were not adjusted for age or smoking [8]. The Nurses' Health Study reported that women who had undergone bilateral oophorectomy showed an increased risk of coronary heart disease compared to premenopausal women (rate ratio, 2.2; 95 percent confidence limits, 1.2 and 4.2) [9]. Interestingly, recent studies have reported elevated risk factors for cardiovascular disease including body fat composition [10], lipids and lipoprotein [11], and vascular remodeling [12-13] in women transitioning through the menopause. Increased susceptibility to osteoporosis and cardiovascular disease in postmenopausal (see Glossary) women may be associated with changes in their hormone levels.”

4) The role of FSH in gonadal development and beyond

a. Second paragraph, pg 3 - also need to state that other investigators have not been successful in reproducing the original experiments conducted in HUVECs (e.g., Stelmaszewska et al did not find FSHR expression in HUVECs, nor did FSH treatment show effects on tube formation, NO production, wound healing or proliferation in HUVECs.)

Authors' response: We thank the reviewer for their comments. We are sorry for not including these information in our original manuscript due to the limit of references numbers requested by the Journal. We have included new text in our revised manuscript (Page 4, line 113-118).

“A number of reports have recently demonstrated that it is also expressed in a range of extragonadal sites, including human osteoclasts [31] and monocytes [32], tumor vasculature and metastases [33], the liver [34] and human umbilical vein endothelial cells (HUVECs) [35, 36], although other independent investigators have not been successful in reproducing the original experiments conducted in HUVECs [37].”

b. Second paragraph, pg 3, 4th sentence from the bottom "The decline in estrogen across the period of menopausal transition..." needs references to support this statement.

Authors' response: References have been added into our revised manuscript [ref 38, 39] (page 5, line 123).

5) FSHR mutations and their clinical consequences (page 4 and table 1)

a. This section should be linked to extragonadal tissue response, pathophysiology/disease, if not, suggest that this should be removed.

b. Table 1 - link the "Phenotype of subjects" column with extragonadal consequences of these phenotypes.

c. Line 8 - "have demonstrated that each motif plays an important role in FSHR function...(box2)..." The description in Box 2 describes FSHR signaling cascades, but does not appear to discuss how different mutations impact signaling through these cascades. Please clarify.

Authors' response: We thank again for the reviewer's comments. All the functional studies for these mutations in this section were performed in HEK293 cells or granulosa cells. These mutations are not associated with extragonadal tissue response, pathophysiology/disease, therefore we have removed this section for our revised manuscript.

6) FSH enhances bone resorption in postmenopausal women (page 5)

a. This subheading is somewhat misleading as the evidence presented does not actually detail how FSH enhances bone resorption in postmenopausal women per se. A more appropriate subheading could be "FSH and the risk for osteoporosis"

Authors' response: We agree with the reviewer's comments. We have amended the subheading for this section (page 5, line 129).

"FSH and the risk for osteoporosis"

b. Paragraph 1, Line 1 - "Osteoporosis is characterized by an absolute reduction in BMD and an associated risk for bone fractures" Reference needed.

Authors' response: The reference has been added in our revised manuscript [ref 3] (page 2, line 79).

c. Paragraph 1, Line 3 - "Estrogen deficiency has [long] been considered [for long] as a major contributor of postmenopausal osteoporosis" Also needs reference.

Authors' response: We have amended our text (page 5) and included the reference in our revised manuscript [ref 40] (page 5, line 132).

"Estrogen deficiency has long been considered as a major contributor of postmenopausal osteoporosis [40]."

d. Paragraph 1, Line 4 - The sentence that being with "However, studies have revealed that nearly half..." is unclear. This sentence needs to be restructured and/or peri-menopause needs to be better defined in the Glossary. This text refers to Box 3, which describes the hormonal alterations in estrogen and FSH across the menopausal transition, but including this information in the introduction with a figure (that is referred to here) would be more clear.

Authors' response: We have included an introduction section and a figure for our revised manuscript (page 3-4, line 61-96). We have restructured the sentences (page 5, line 132-137) and re-defined peri-menopause in Glossary (page 21, line 12).

"However, it has been reported that bone loss occurs around 2–3 years before the FMP [41], when estrogen levels are relatively normal while FSH levels are significantly elevated (Figure

1). In addition, previous cross-sectional [42] and longitudinal [43] studies have shown that higher serum FSH levels but not lower serum estradiol levels are associated with lower bone mineral density among pre- and early perimenopausal women enrolled in SWAN.”

Glossary

“Perimenopause (STRAW +10 Staging System: stage -2 to +1a): the time around menopause and begins at Stage –2 and ends 12 months after the final menstrual period (Stage +1a).”

e. Paragraph 2 - When citing references 45 and 47, the statements do not indicate what the outcomes may be adjusted for (such as education, physical activity level, diet, etc.). These adjustments are detailed in the results sections of the references papers.

Authors’ response: We thank the reviewer for consideration of this point. We have amended our text to include these adjustments (page 5-6, line 142-155).

“A small cohort study has shown that women with hypergonadotropic amenorrhea (mean FSH>40 mIU/mL) have lower lumbar spine bone density than those with hypogonadotropic amenorrhea (mean FSH<40 mIU/mL), with comparable estrogen levels between the two groups. In addition, only FSH levels were negatively correlated with lumbar spine bone density in hypergonadotropic amenorrheic women, after adjustment for age and body mass index (BMI) [44]. Furthermore, previous studies investigated 2,375 pre and early perimenopausal women from SWAN and found that higher FSH concentrations were associated with elevated concentrations of the bone resorption marker urinary N-telopeptide of type I collagen (NTx), after adjustment for BMI, thyroid disease, insulin sensitivity, difficulty in paying for basics, race/ethnicity, seasonality and site. Higher FSH concentrations were also associated with higher osteocalcin concentrations (bone formation maker), after adjustment for smoking, physical activity, physical functioning, BMI, diabetes, insulin sensitivity, thyroid disease, race and site [45].”

f. Paragraph 2, line 5 - Sentence that begins with "Interestingly, postmenopausal women with an activating FSHR polymorphism..." seems out of place in this section. Would make more sense in the section about FSHR mutations. This is also an example of what could be included in the FSHR mutation section to link the mutation with extragonadal consequences.

Authors’ response: We have removed the FSHR mutation section in our revised manuscript.

g. Paragraph 2, line 6 - Sentence that begins with "Furthermore, a cross-sectional study of 2,375 peri-menopausal women..." is misleading. Authors state evidence for bone turnover, but only present data regarding bone resorption. Were bone formation markers measured and reported in the original paper? If so, they need to be included in this sentence or bone turnover needs to be changed to bone resorption.

Authors’ response: We are sorry for not including bone formation markers in our original manuscript. We have amended the text in our revised manuscript (page 5-6, line 147-155).

“Furthermore, previous studies investigated 2,375 pre and early perimenopausal women from

SWAN and found that higher FSH concentrations were associated with elevated concentrations of the bone resorption marker urinary N-telopeptide of type I collagen (NTx), after adjustment for BMI, thyroid disease, insulin sensitivity, difficulty in paying for basics, race/ethnicity, seasonality and site. Higher FSH concentrations were also associated with higher osteocalcin concentrations (bone formation maker), after adjustment for smoking, physical activity, physical functioning, BMI, diabetes, insulin sensitivity, thyroid disease, race and site [45].”

h. Paragraph 2, line 10 - Sentence beginning with "Further studies have shown that FSH levels are positively correlated with bone resorption..." only includes data for CTX, but the paper also reported osteocalcin. Why was only CTX included in this statement? This sentence might also better fit in the next paragraph since that paragraph focuses on the imbalance between formation and resorption. Also the end of the sentence states "postmenopausal healthy women and menopausal transition women" these phrases need to be edited for word order and correct term use.

Authors’ response: We thank the reviewer for their comments. We agree with the reviewer that it fits better in the next paragraph. We have amended our text and moved it to the next paragraph (page 6-7, line 180-185).

“Interestingly, further studies have shown that serum levels of FSH are positively correlated with the bone resorption marker CTX and the bone formation marker osteocalcin in healthy postmenopausal women [57], indicating a potential role of FSH in bone turnover. In addition, a significantly positive association between FSH and CTX has also been reported in osteoporotic Chinese women during the menopausal transition [58].”

i. Paragraph 3, line 1 - needs reference

Authors’ response: The reference has been added [ref 49] (page 6, line 164).

j. Paragraph 3, line 8 - what is meant by "conceptive studies"?

Authors’ response: We are sorry for the mistake. We have amended our text (page 6, line 176).

“several studies have been performed to test whether the specific inhibition of FSH can prevent ovariectomy-induced bone loss in mouse models.”

k. Paragraph 3 - References 58 and 59 are from mice, but this is not explicitly stated. Please indicate that this is not human data.

Authors’ response: We thank the reviewer for consideration of this point. We have clearly stated these data are from mice in our revised manuscript (page 6, line 176-180).

“several studies have been performed to test whether the specific inhibition of FSH can prevent ovariectomy-induced bone loss in mouse models. Indeed, blocking FSH with a specific antibody targeting its β -subunit increases bone mass and reduces body fat in mouse models [55, 56].”

7) FSH increases the risk of cardiovascular disease in postmenopausal women

a. We suggest that the heading be changed to "FSH and the risk for cardiovascular disease"

Authors' response: We have amended the subheading title for this section (page 7, line 188).

“FSH and the risk for cardiovascular disease”

b. Paragraph 1, line 4 - The section of this paragraph starting with "Interestingly, epidemiological studies have shown that estrogen replacement therapy..." is confusing and the transition 'however' appears to be misused. The clinical trial data showed harm in women who were 20+ years postmenopause.

Authors' response: We thank the reviewer for their comments. We have amended the text in our revised manuscript (page 7, line 192-200).

“Whether estrogen replacement therapy could reduce cardiovascular disease remains ambiguous. Previous studies have shown that estrogen replacement therapy at standard daily dose of 0.625mg or less decreases cardiovascular disease risk in postmenopausal women without previous history of heart disease. However, estrogen at a higher daily dose of 0.625mg or more and in combination with progestin may modestly increase the risk for stroke [60, 61]. Recent clinical trials have demonstrated that menopausal hormone therapy for 5 to 7 years is not associated with risk of all-cause, cardiovascular, or cancer mortality among 27,347 postmenopausal women aged 50 to 79 years during a cumulative follow-up of 18 years [62].”

c. Paragraph 1, line 9 - If a figure showing the FSH/estrogen changes over the menopausal transition is included, it could be referenced at the end of the sentence starting with "In contrast to the decline in estrogen levels observed during menopause.."

Authors' response: We have included figure 1 in our revised manuscript and referred to it here (page 7, line 202).

d. Paragraph 1, line 13 - Include what the outcomes were for determining "subclinical atherosclerosis" (e.g., coronary artery calcium, carotid IMT).

Authors' response: We have amended our text and include these information (page 7, line 203-206).

“Several previous studies have examined associations between FSH levels and subclinical atherosclerosis including coronary artery calcium and the carotid intima-media thickness (IMT) in women, with the majority of them reporting a positive correlation [63-65].”

e. Paragraphs 2 & 3 - The mechanisms linking FSH and CVD need to be more clearly organized/listed so that it is easy to read. We suggest breaking it into paragraphs or sections that focus on adiposity, angiogenesis, lipids, calcification, and endothelial activation with a bit more detail in each section. Right now, the section on endothelial cell activation is highly detailed, while just a sentence or two are dedicated to each other mechanism listed. If the preference of the

authors is to keep the emphasis on endothelial cells as a primary mechanism, then we suggest moving paragraph 3 ahead of paragraph 2.

Authors' response: We thank the reviewer for their suggestions and we agree this section should be restructured. We have broken this section into several paragraphs (page 8-10, line 218-280).

“Potential mechanisms by which FSH increases risk of cardiovascular disease

FSH may increase risk of cardiovascular disease through a number of distinct mechanisms (Figure 4):

Effects of FSH on endothelial cell activation and atherosclerosis

Endothelial cell activation by oxidized lipids and pro-inflammatory stimuli plays an important role in the initiation and progression of atherosclerosis. Activated endothelial cells express a variety of adhesion molecules such ICAM-1 and VCAM-1, which recruits leukocytes to the endothelium [68]. Our recent report demonstrated for the first time that FSH directly accelerates atherosclerosis in ovariectomized ApoE^{-/-} mice, independent of estrogen deficiency. Mechanistically, FSH up-regulated VCAM-1 expression in human umbilical vein endothelial cells and subsequently increased human monocyte adhesion through FSHR/Gαs/cAMP/PKA and PI3K/Akt/mTOR/NF-κB signaling pathways. Interestingly, FSHR was specifically localized in caveolae of endothelial cells. Caveolin-1, the main protein component of caveolae, directly interacted with FSHR and mediated its downstream signaling actions [36].

FSH accelerates adiposity and lipid accumulation

Cui *et al* recently reported that FSH treatment increases the weight of abdominal fat and lipid accumulation of adipocytes in chickens, through enhancing the expression of genes in the fatty acid metabolism pathway, retinol metabolism pathway, and peroxisome proliferator-activated receptor (PPAR) signaling pathway [69]. Further studies showed that FSHR was functionally expressed in human and mouse fat tissues and adipocytes. FSH treatment increased lipid biosynthesis, lipid droplet formation, altered the secretion of leptin and adiponectin through the Gαi/Ca²⁺/CREB pathway in mouse 3T3-L1 preadipocytes [70]. Interestingly, recent elegant studies performed by Liu *et al* have shown that blockade of FSH signaling with a specific antibody reduces high-fat diet-induced obesity in wild type mice and adiposity in ovariectomized mice [55]. Specifically, antibody-treated mice showed significantly reduced total (TFV), subcutaneous (SFV) and visceral fat volume, but interscapular brown adipose tissue remained unchanged [55]. These data suggest that FSH may play an important role in determining body fat distribution, which may contribute to cardiovascular dysfunction [71]. Interestingly, it has been shown that serum FSH levels are positively associated with serum total cholesterol and LDL-C levels in Chinese postmenopausal women [34]. FSH administration in ovariectomized mice promotes dyslipidemia through inhibiting hepatic cholesterol metabolism. Mechanistically, FSH reduces LDLR levels in hepatocytes and attenuates the endocytosis of LDL-C, leading to an elevated circulating LDL-C level [34], which is an independent risk factor for atherosclerosis. Taken together, these studies suggest that FSH may increase risk factors for cardiovascular disease through accelerating adiposity and lipid accumulation.

FSH promotes angiogenesis

Angiogenesis inhibition through the application of endostatin or TNP-470 has been shown to decrease plaque formation and intimal neovascularization in ApoE^{-/-} mice [72]. FSH signaling through FSHR in HUVECs promotes angiogenesis, which is independent of VEGF [73]. Interestingly, treatment of HUVECs with FSH stimulated the PI3K/AKT signaling pathway, with similar efficacy as VEGF [73]. These data suggest that FSH may increase atherosclerosis through promoting angiogenesis. However, the effect of FSH on angiogenesis cannot be reproduced by other investigators and the discrepancy remains to be clarified [37].

Potential role of FSH in vascular calcification.

Vascular calcification is a common feature of advanced atherosclerosis and is important in determining atherosclerotic plaque stability [74]. We and others have previously demonstrated that vascular calcification shares many similarities to bone remodeling, which involves maintaining a balance between osteoblasts and osteoclasts [75-77]. The presence of osteoclast-like cells and active resorption of ectopic mineral deposition have been reported in calcified atherosclerotic plaques [78]. Furthermore, monocytes and macrophages are frequently observed in atherosclerotic plaque [79]. These data suggest that osteoclasts, through a signaling cascade involving FSH (Figure 3) may be recruited to calcified regions where they can resorb mineral deposition, subsequently increasing the incidence of plaque rupture [80] (Figure 4). In addition, FSH stimulates immune cells to release inflammatory cytokines [53, 54], which may also contribute to vascular calcification (Figure 4). ”

f. Paragraphs 2 & 3 - Check this section to make sure it is clearly state which results are from clinical versus mouse versus cell studies.

Authors’ response: We thank the reviewer for their comments. We have clearly indicated which results are from clinical versus mouse versus cell studies in our revised manuscript.

g. Paragraph 2 - Although increased adiposity is touched on briefly as a potential mechanism for increased CVD risk, the importance of body fat distribution is not addressed. The referenced study by Liu et al. reported reduced visceral adiposity after FSH antibody treatment in mice, but this finding is not included. Considering the potential effect of FSH in determining body fat distribution is an important mechanism underlying cardiovascular risk.

Authors’ response: We thank the reviewer for their comments. We have included this point in our revised manuscript (page 9, line242-249).

“Interestingly, recent elegant studies performed by Liu *et al* have shown that blockade of FSH signaling with a specific antibody reduces high-fat diet-induced obesity in wild type mice and adiposity in ovariectomized mice [55]. Specifically, antibody-treated mice showed significantly reduced total (TFV), subcutaneous (SFV) and visceral fat volume, but interscapular brown adipose tissue remained unchanged [55]. These data suggest that FSH may play an important role in determining body fat distribution, which may contribute to cardiovascular dysfunction [71].”

h. Paragraph 2, Line 17 - "Osteoclasts, through a signaling cascade involving FSH (as described above)..." Is this referring to the signaling cascade described in one of the boxes or Fig 1 (I do not see Fig 1 referenced in the text?) The reference to "above" needs clarification.

Authors' response: We are sorry for this confusion. This is referring to the signaling cascade described in Figure 3.

Authors' action: We have amended our text and referred to Figure 3 and Figure 4 (page 9-10, line 270-278).

“We and others have previously demonstrated that vascular calcification shares many similarities to bone remodeling, which involves maintaining a balance between osteoblasts and osteoclasts [75-77]. The presence of osteoclast-like cells and active resorption of ectopic mineral deposition have been reported in calcified atherosclerotic plaques [78]. Furthermore, monocytes and macrophages are frequently observed in atherosclerotic plaque [79]. These data suggest that osteoclasts, through a signaling cascade involving FSH (Figure 3) may be recruited to calcified regions where they can resorb mineral deposition, subsequently increasing the incidence of plaque rupture [80] (Figure 4).”

8) Clinical use and long-term safety on cardiovascular disease of FSH agonists

a. Why does this section heading only refer to CVD risk and not osteoporosis risk? Even if there is a paucity of data, this should be addressed and included in the title.

Authors' response: We thank the reviewer for their comments. We have amended the title for this section (page 10, line 282-283).

“Clinical use and long-term safety of recombinant FSH therapies against cardiovascular disease and osteoporosis”

b. Is FSH agonist the correct term to us to refer to these recombinant FSH therapies?

Authors' response: We agree with the reviewer's comments. We have changed “FSH agonist” to “recombinant FSH therapies” (page 10, line 293).

c. Paragraph 1 - Are all of these details about recombinant FSH therapy necessary?

Authors' response: We thank the reviewer for the suggestion. We have removed all of these details about “recombinant FSH therapies” in our revised manuscript.

d. Paragraph 2 should be moved before Paragraph 1. The entire paper to this point is discussing postmenopausal women, and then this section starts with talking about the different recombinant FSH therapies that are available. The initial thought is, why would a postmenopausal woman need FSH therapy? The second paragraph goes on to explain that these therapies are used in premenopausal women undergoing fertility treatments and disease risk has been assessed in women who have undergone these therapies. It is this paragraph that is of most importance in this section. With this context in mind it is easier to understand the content in this section

Authors' response: We agree with reviewer's comments. We have removed Paragraph 1 in our revised manuscript.

9) Concluding remarks and future perspectives

a. Line 2 - "Despite growing evidence showing that FSH accelerates osteoporosis and CVD in postmenopausal women..." This statement needs to be tempered. There are no direct data in humans that show that FSH accelerates osteoporosis and CVD. There are data that suggest that FSH could contribute to the decline in bone health with the menopause transition. There are no direct data concerning CVD.

Authors' response: We agree with the review's comments. We have amended our text in the revised manuscript (page 11, line 301-307).

"Epidemiological and animal studies have revealed that FSH contributes to the decline in bone health in women undergoing menopausal transition. Emerging data has shown that FSH treatment enhances atherosclerotic plaque formation in ovariectomized AopE^{-/-} mice [36]. However, our understanding of the molecular mechanisms through which FSH increases the risk of osteoporosis and cardiovascular disease is far from complete (see outstanding questions)."

b. Line 5 - "Particularly, most research investigating the effects of FSH on [postmenopausal] osteoporosis and CVD has focused on a limited number of FSH target genes..." Where are these data presented in the paper? By target genes are the authors referring to signaling pathways?

Authors' response: We thank the reviewer for their comments. We have amended the text in our revised manuscript (page 11, line 307-309).

"The vast technological advancements such as Next-Generation Sequencing will provide valuable new information on the molecular pathophysiology of FSH in extragonadal tissues, such as bone and blood vessels."

c. Line 11 - "In addition, blockade of FSH has been shown, at least in mice, to improve postmenopausal osteoporosis and CVD." This statement needs to be reworded. Mice do not undergo menopause, so stating it as improve OVX induced bone loss (or a model of menopause) would be more appropriate. Also, please present and reference the data that FSH blockage improves CVD in mice.

Authors' response: We thank the reviewer for their comments. We have previous shown that FSH treatment enhances atherosclerosis in ovariectomized AopE^{-/-} mice [36]. We have amended the text in our revised manuscript (page 11, line 309-318).

"Animal studies have shown that blocking FSH increases bone mass, reduces body fat and induces thermogenic adipose tissue. These data suggest that blocking FSH may not only be a potential opportunity for treating osteoporosis, but also of benefit in the treatment of visceral fat associated diseases such as metabolic syndrome and cardiovascular disease [55]. Whether FSH promotes osteoporosis and cardiovascular disease in women remains to be further elucidated in humans. In conclusion, a fuller understanding of the molecular pathological roles of FSH for osteoporosis and cardiovascular disease in postmenopausal women could

have significant importance in improving diagnosis, management and treatment of these major diseases.”

10) Box 1

a. Line 3 - "FSH levels are strictly controlled by the HPG axis and [the steroid] sex hormone[s]- mediated feedback" This needs rewording

Authors’ response: We have amended our text (page 19, box1, line 3-4).

“FSH levels are strictly controlled by the hypothalamic pituitary gonadal axis and sex hormone-mediated negative feedback.”

b. Line 6 - "produce FH and LH, which exert[s] their primary function..." Remove the s in exerts.

Authors’ response: We have amended our text (page 19, box1, line 6).

“which exert their primary function in the gonads.”

c. The glycosylation issue is a potentially very important one, particularly in extragonadal tissues. May want to consider mentioning this in the main text of the article.

Authors’ response: We thank the reviewer for the suggestion. We have mentioned this in our main text of the article (page 4, line 104-105).

“Similar to other glycoprotein hormones, glycosylation of FSH plays an important role in regulating its activity [15] (see Box1).”

11) Box 2

a. Are the signaling cascades mentioned in this box pertinent to specific extragonadal tissues? Are some specific to certain cell types in bone or the cardiovascular system? IF so, this should be mentioned?

Authors’ response: We thank the reviewer for their comments. These signaling cascades are not pertinent to specific extragonadal tissues and not specific to certain cell types in bone and the cardiovascular system. These signaling cascades were reported in granulosa cells, Sertoli cells or HEK293 cells. Some of these signaling pathways such as ERK, AKT have also been reported in extragonadal cells, such as HUVECs, preadipocytes etc.

b. There is a lot of detail in here without much context and the long list of Gas-independent mechanisms in the middle of the box are hard to follow. Are these pathways stimulated through FSH interaction with FSHR or are they alternative signaling pathways for FSH outside of FSHR? Without referring out to the cited references, it is difficult to follow the importance of the included information. Would an accompanying figure lend more relevance?

Authors’ response: We thank the reviewer for their suggestions. These signaling pathways are stimulated via FSH interaction with FSHR coupled with multiple different partners. We have amended our text and provided a new figure for FSH downstream signaling pathways (box2, page 20).

“It is now well recognized that upon FSH binding, the activated FSHR triggers a number of intracellular signaling pathways either in parallel or sequentially. Classically, upon FSH activation, FSHR functionally couples to *G_s* subunit and induces the cAMP/PKA signaling pathway, subsequently activating the cAMP regulatory element-binding protein (CREB) and modulating gene expression, such as aromatase and inhibin- α [24]. This canonical signaling pathway plays a key role for its biological actions inside granulosa cells in the ovary and sertoli cells in the testes. It is now clear that FSHR also involves other transduction mechanisms upon FSH binding. In recent years, a number of *G_s*-independent transduction mechanisms have been found. It has been reported that FSHR also couples to *G_i* [25], *G_q* [26], β -arrestins [27], Adapter protein containing the Pleckstrin homology domain, the Phosphotyrosine binding domain, and the Leucine zipper motif (APPL) [28-30]. These FSHR interacting partners enable FSH to activate alternative signaling pathways, including those mediated by distinct kinases such as inositol trisphosphate (IP3), Akt and ERK1/2. This complex signaling network triggered by FSH fine-tunes its biological action and determines the fate of target cells (Figure 2).”

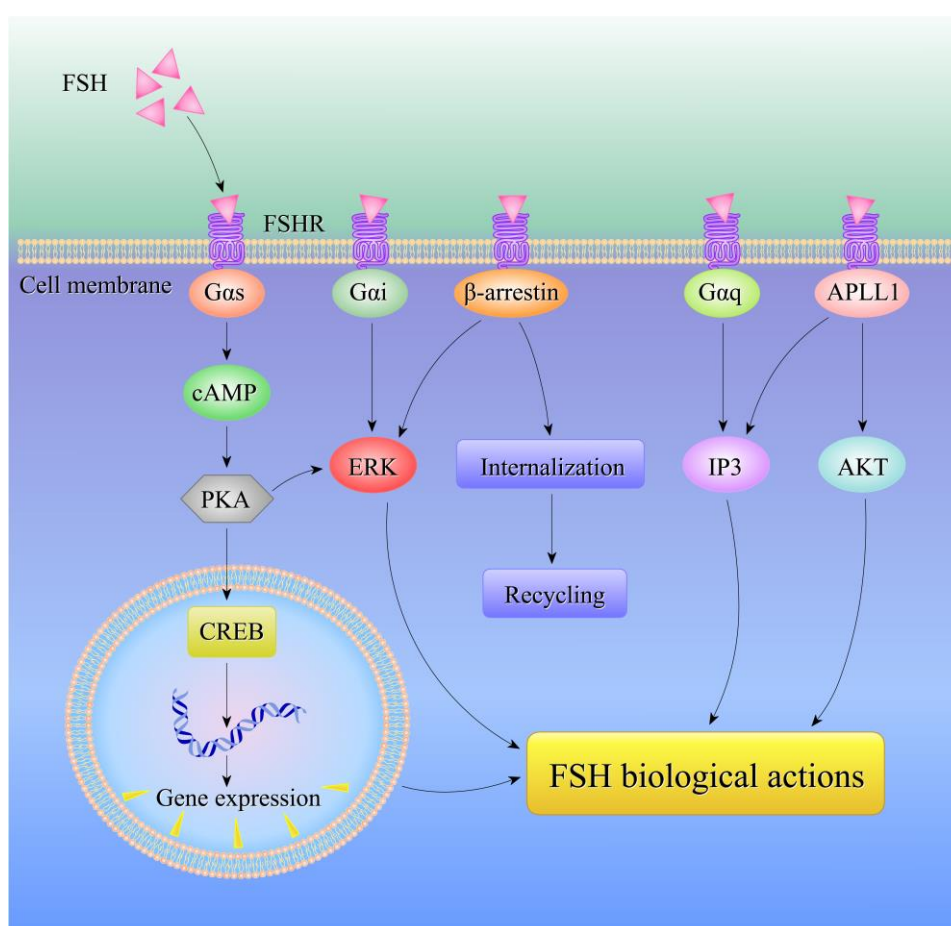


Figure 2. Multiple intracellular signaling pathways induced by FSH. Classically, upon FSH binding, the activated FSHR induces canonical *G_s*/cAMP/PKA signaling pathway, which subsequently activates CREB and regulates target gene expression. FSHR also activates its downstream signaling pathways through other *G_s*-independent transduction mechanisms mainly coupling to other *G α* subunits, β -arrestin-dependent signaling, EGFR transactivation, and APPL1-mediated signals [20-26].

12) Box 3

a. This information would fit nicely into an introduction section with an accompanying figure.

Authors' response: We agree with the reviewer's comments. We have added an introduction section with Figure 1 for our revised manuscript (page 3-4, line 61-96).

b. The description of the SWAN study is longer than necessary. Although details about the SWAN study are pertinent, including more detailed results instead of just two spot measures of E2 and FSH would be informative. Particularly, detailing the time course of the change of both hormones over the menopausal transition would be helpful.

Authors' response: We agree with the reviewer's comments. We have added an introduction and Figure 1 in our revised manuscript.

13) Table 1

a. Remove unless specific connections with extragonadal tissues (osteoporosis/CVD) are made

Authors' response: It has been removed in our revised manuscript.

14) Glossary

a. Including menopause, perimenopause, postmenopausal and the menopause transition is redundant and the definitions provided are unacceptable. The STRAW+10 criteria should be used and cited for all definitions (Harlow SD et al. Menopause. 2012 Apr;19(4):387-95.)

Authors' response: We thank the reviewer for their constructive advice. We have re-defined these terms using The STRAW+10 criteria (page 21, Glossary).

“Menopausal transition (STRAW +10 Staging System: Stages -2 to -1) [ref. 1]

Early menopausal transition (Stage -2): Criteria include increased variability in menstrual cycle length, a persistent difference of 7 days or more in the length of consecutive cycles, elevated but variable FSH levels, low antimüllerian hormone and antral follicle count.

Late menopausal transition (Stage -1): Criteria include the occurrence of amenorrhea of 60 days or longer, elevated FSH levels greater than 25 IU/L, low antimüllerian hormone and antral follicle count, and vasomotor symptoms (likely).

Perimenopause (STRAW +10 Staging System: stage -2 to +1a): the time around menopause and begins at Stage -2 and ends 12 months after the final menstrual period (Stage +1a).

Postmenopause (STRAW +10 Staging System: Stage +1a, +1b, +1c, +2): The time after the final menstrual period.”

15) Figure 1

a. Legend - "RANKL released by osteoblasts or T cells binds to RANK in monocytes and promotes osteoclast formation [which subsequently leads to osteoporosis]" The end of this sentence (in brackets) needs to be revised with more detail. Oversimplification.

Authors' response: We thank the reviewer for their comments. We have amended the figure legend with more detail for [which subsequently leads to osteoporosis] (page 22, line 570-578).

“Figure 3. FSH accelerates bone resorption through increasing osteoclast formation. FSH directly enhances RANK expression in monocytes [50-52]. RANKL released by osteoblasts or T cells binds to RANK in monocytes and promotes osteoclasts formation. FSH also stimulates monocytes and macrophages to release inflammatory cytokines such as TNF α , IL1 β and IL6 [53-54]. These inflammatory cytokines stimulate osteoblasts and T cells to secrete RANKL, which promotes osteoclasts formation. Osteoclasts are the cells responsible for bone resorption, and osteoblasts are the cells for bone formation. Increased ratio of osteoclasts to osteoblasts leads to bone mineral loss, which may subsequently cause osteoporosis [49].”

b. Line 4 - The use of the transition "conversely" appears to be incorrect. It should state, "FSH also stimulates..." because it is still describing a mechanism leading to bone resorption.

Authors' response: We agree the reviewer's comments. We have amended our text accordingly (page 22, line 573-574).

“FSH also stimulates monocytes and macrophages to release inflammatory cytokines such as TNF α , IL1 β and IL6 [50-51].”

c. The use of references may be appropriate in the figure legend.

Authors' response: We have included references in our figure legend (page 22).

16) Figure 2

a. Line 1 - "FSH increases the expression of VCAM-1" is this direct or through some other mechanism? On the figure there is no arrow directly from FSH to VCAM-1, this should be added if it is a direct effect.

Authors' response: We thank the reviewer for their comments. We have previously shown that FSH directly increases the expression of VCAM-1 [ref 36 in the main text]. The arrow has been added in Figure 4.

b. Line 7 - T cells are mentioned as a cell type that FSH may stimulate to release inflammatory cytokines, but they are not included on the figure.

Authors' response: T cells have been added in Figure 4.

c. The use of references may be appropriate in the figure legend.

Authors' response: We have included references in our figure legend (page 22).

17) Outstanding questions

a. #3 - Suggest rewording to state: Do osteoclast or vascular endothelial cell specific FSHR conditional knockout mice demonstrate improved bone and vascular health?

Authors' response: We thank the reviewer for their suggestions. We have amended our text in the revised manuscript (outstanding questions, line 7-8).

“Do osteoclast or vascular endothelial cell specific FSHR conditional knockout mice demonstrate improved bone and vascular health?”

b. #4 - "FSH enhanced postmenopausal atherosclerosis" This statement is reaching and should be reworded. There is no direct evidence to support this in postmenopausal women.

Authors' response: We agree with the reviewer's comments. We have amended our text in the revised manuscript (outstanding questions, line 9-10).

"Are there any other mechanisms involved in FSH enhanced atherosclerosis in ovariectomized ApoE^{-/-} mice such as induction of inflammation or foam cell formation?"

c. #5 - Needs to be edited for word order.

Authors' response: We have amended our text in the revised manuscript (outstanding questions, line 11-12).

"FSH blocking increases bone mass and reduces body fat in mouse models. Can these findings be translated into humans?"

d. Other suggested outstanding questions not mentioned but could be included:

i. Potential for FSH actions outside of FSHR

ii. Importance of glycosylation state in FSH action in extragonadal tissues

Authors' response: We thank the reviewer for their suggestions. We have added these two points in our revised manuscript (outstanding questions, line 13-15).

"Are there any potential mechanisms for FSH biological activities without binding to FSHR in extragonadal tissues?"

What are the effects of glycosylation state of FSH on its actions in extragonadal tissues?"

Highlights:

Epidemiological studies have demonstrated that FSH levels are positively associated with postmenopausal osteoporosis and cardiovascular disease.

Besides gonadal tissues, FSHR is found to be expressed by a range of extragonadal sites, including osteoclasts and endothelial cells.

FSH treatment stimulates osteoclast cell formation, function and survival, which are the cells responsible for bone resorption. In addition, FSH administration significantly promotes atherosclerosis in ovariectomized ApoE^{-/-} mice by up-regulating VCAM-1 expression in human umbilical vein endothelial cells and subsequently increasing human monocyte adhesion.

Blockade of FSH with a specific antibody targeting its β -subunit enhances bone mass, reduces body fat and atherosclerosis in mouse models.

FSH represents a potential novel therapeutic target for the treatment of postmenopausal osteoporosis and cardiovascular disease.

Extragenadal effects of follicle-stimulating hormone on osteoporosis and cardiovascular disease in women with menopausal transition

Short title: The pathological roles of FSH in women with menopausal transition

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Keywords:

Follicular Stimulating Hormone/Postmenopause/Osteoporosis/Cardiovascular disease/Extragenadal effects

ABSTRACT:

The risk of osteoporosis and cardiovascular disease increases significantly in postmenopausal women. Until recently, the underlying mechanisms have been primarily attributed to estrogen decline following the menopause. However, FSH levels rise sharply during menopausal transition and are maintained at elevated levels for many years. FSHR has been detected in various extragonadal sites, including osteoclasts and endothelial cells. Recent advances show that FSH may also contribute to postmenopausal osteoporosis and cardiovascular disease. We review here the key actions through which FSH could contribute to the development of osteoporosis and cardiovascular disease in women as they transition through the menopause. Advancing our understanding of the precise mechanisms through which FSH promotes osteoporosis and cardiovascular disease may provide new opportunities for improving quality of life for postmenopausal women.

Introduction

During menopausal transition, women undergo irregular menstruation or infrequent menses, with the permanent cessation of menstrual cycle due to depletion of viable follicles [1]. The length of menopausal transition is highly variable, ranging from 3-9 years in women aged around 45–55 years old. Menopausal transition is associated with alterations in hormone profiles. The two well established hormonal profiles are (1) a marked decline in estradiol and (2) a notable rise in follicle-stimulating hormone (FSH). The Study of Women's Health Across the Nation (SWAN), the most comprehensive U.S. study to date, followed premenopausal women over the menopause transition. Previous studies examined 1,215 SWAN participants who transitioned through the menopause over time and reported that FSH levels increased as early as 6 years before the final menstrual period (FMP) and stabilized 2 years after the FMP. Estrogen levels declined until 2 years before the FMP and stabilized after 2 years around the FMP [2] (Figure 1). Across the menopausal transition, serum levels of FSH increased from 15.15 mIU/mL to 98.21 mIU/mL, while serum levels of estradiol dropped from 60.26 pg/ml to 19.12 pg/ml [2].

Osteoporosis is defined as a skeletal disease with a significant reduction in bone mineral density and an associated increased risk of bone fracture [3]. Menopause is widely recognized as a risk factor for osteoporosis and bone fracture in women's later life. It has been reported that bone loss accelerates during the late stage of menopausal transition and continues into old age [4-6]. A large cohort of UK women study has shown that the relative risk (RR) of hip fracture in postmenopausal women is significantly higher than in premenopausal women with similar age (adjusted RR 2.22, 95% confidence interval [CI] 1.22–4.04; $p=0.009$) [7]. Menopause is also thought to be a risk factor for cardiovascular disease in women. The Framingham Heart Study reported that postmenopausal women aged 50–59 years had 4 times the 10-year incidence of coronary heart disease as premenopausal women of similar age, but results were not adjusted for age or smoking [8]. The Nurses' Health Study reported that women who had undergone bilateral oophorectomy showed an increased risk of

coronary heart disease compared to premenopausal women (rate ratio, 2.2; 95 percent confidence limits, 1.2 and 4.2) [9]. Interestingly, recent studies have reported elevated risk factors for cardiovascular disease including body fat composition [10], lipids and lipoprotein [11], and vascular remodeling [12-13] in women transitioning through the menopause. Increased susceptibility to osteoporosis and cardiovascular disease in **postmenopausal** (see Glossary) women may be associated with changes in their hormone levels.

The role of FSH in gonadal development and beyond

FSH belongs to the pituitary glycoprotein hormone family, which also includes luteinizing hormone (LH), chorionic gonadotropin and thyroid-stimulating hormone. These four hormones share a common α subunit, which forms a functional heterodimer with a hormone-specific β subunit. FSH primarily exerts its biological function through binding to the FSH receptor (FSHR), which is predominantly expressed by granulosa cells in the ovary and sertoli cells in the testes, respectively [14]. **Similar to other glycoprotein hormones, glycosylation of FSH plays an important role in regulating its activity [15] (see Box1).** Classically, FSH plays an essential role in gonadal development, maturation and function. In females, it promotes growth and maturation of ovarian follicles and estrogen production, whereas in males it supports spermatogenesis [23]. **Through binding to FSHR coupled with different partners, FSH activates multiple downstream signaling pathways (see Box2, Figure 2).**

Expression of FSHR determines the targets and extent of its biological action. Traditionally, FSHR is considered to be gonad-specific. **A number of reports have recently demonstrated that it is also expressed in a range of extragonadal sites, including human osteoclasts [31] and monocytes [32], tumor vasculature and metastases [33], the liver [34] and human umbilical vein endothelial cells (HUVECs) [35, 36], although other independent investigators have not been successful in reproducing the original experiments conducted in HUVECs [37].** These data have prompted researchers to study the extragonadal effects of FSH and in particular its potential actions in

postmenopausal women, who show increased risk of osteoporosis and cardiovascular disease. The decline of estrogen across the period of **menopausal transition** has previously been considered as a major contributor of postmenopausal osteoporosis and cardiovascular disease [38, 39], however recent studies suggest that FSH may also play an important role. In the present review, we will discuss the latest insights into the potential pathological roles of FSH in menopausal women that exceed its classical gonadal regulatory role and examine the underlying mechanisms through which FSH may promote osteoporosis and cardiovascular disease.

FSH and the risk of osteoporosis

Postmenopausal women are the majority of patients who suffer from osteoporosis. Estrogen deficiency has long been considered as a major contributor of postmenopausal osteoporosis [40]. However, it has been reported that bone loss occurs around 2–3 years before the FMP [41], when estrogen levels are relatively normal while FSH levels are significantly elevated (Figure 1). In addition, previous cross-sectional [42] and longitudinal [43] studies have shown that higher serum FSH levels but not lower serum estradiol levels are associated with lower bone mineral density among pre- and early perimenopausal women enrolled in SWAN. Thus, estrogen deficiency cannot fully explain postmenopausal osteoporosis and FSH may play additional roles in bone loss during menopause.

Several observational studies have confirmed the association between FSH levels and bone metabolism. A small cohort study has shown that women with hypergonadotropic amenorrhea (mean FSH>40 mIU/mL) have lower lumbar spine bone density than those with hypogonadotropic amenorrhea (mean FSH<40 mIU/mL), with comparable estrogen levels between the two groups. In addition, only FSH levels were negatively correlated with lumbar spine bone density in hypergonadotropic amenorrheic women, after adjustment for age and body mass index (BMI) [44]. Furthermore, previous studies investigated 2,375 pre and early perimenopausal women from SWAN and found that higher FSH concentrations were associated with elevated concentrations of the bone

resorption marker urinary N-telopeptide of type I collagen (NTx), after adjustment for BMI, thyroid disease, insulin sensitivity, difficulty in paying for basics, race/ethnicity, seasonality and site. Higher FSH concentrations were also associated with higher osteocalcin concentrations (bone formation maker), after adjustment for smoking, physical activity, physical functioning, BMI, diabetes, insulin sensitivity, thyroid disease, race and site [45]. A significant association between bone loss and high serum FSH has also been reported in Chinese women [46, 47]. Unexpectedly, Gourlay *et al* have reported that neither FSH nor estrogen shows a strong correlation with bone mass in younger postmenopausal women [48]. These data may be due to the relative small size of patient cohorts and/or study designs. Taken together, these data suggest that FSH is a marker for bone loss in women during menopausal transition, and may directly promote bone resorption.

The underlying mechanisms through which osteoporosis occurs is primarily determined by an imbalance between bone formation and bone resorption [49]. Osteoclasts are the cells responsible for bone resorption. Sun *et al* reported increased bone mass and decreased osteoclastic resorption in haploinsufficient *FSHβ^{+/-}* mice with normal ovarian function yet 50% reduction in serum FSH levels [50], suggesting that the effect of FSH on bone is estrogen independent. Further studies reported that FSH activates MEK/Erk, NF-κB, and Akt signaling pathways through a Gi2α protein-coupled FSHR and directly accelerates osteoclast formation, function and survival [50-52]. Additionally, FSH also indirectly stimulates osteoclast formation by inducing inflammatory cytokine release by immune cells, such as IL-1β, IL-6 and TNF-α [53, 54] (Figure 3). Indeed, Iqbal *et al* found that FSH stimulated bone marrow granulocytes and macrophages to release TNF-α, which expanded the number of bone marrow osteoclast precursors and enhanced osteoblast formation, subsequently leading to the high turn-over bone loss [53]. Having noted the potential role of FSH in postmenopausal bone loss, several studies have been performed to test whether the specific inhibition of FSH can prevent ovariectomy-induced bone loss in mouse models. Indeed, blocking FSH with a specific antibody targeting its β-subunit increases bone mass and reduces body fat in mouse models [55,

56]. Interestingly, further studies have shown that serum levels of FSH are positively correlated with the bone resorption marker CTX and the bone formation marker osteocalcin in healthy postmenopausal women [57], indicating a potential role of FSH in bone turnover. In addition, a significantly positive association between FSH and CTX has also been reported in osteoporotic Chinese women during the menopausal transition [58]. These studies suggest that targeting FSH may be a potential therapeutic target for the clinical treatment and management of postmenopausal osteoporosis and obesity.

FSH and the risk for cardiovascular disease

Until recently, it has been widely accepted that the risk of cardiovascular disease significantly increases in postmenopausal women, with the reduction in estrogen levels associated with menopausal transition considered to be a major contributor [59]. Whether estrogen replacement therapy could reduce cardiovascular disease remains ambiguous. Previous studies have shown that estrogen replacement therapy at standard daily dose of 0.625mg or less decreases cardiovascular disease risk in postmenopausal women without previous history of heart disease. However, estrogen at a higher daily dose of 0.625mg or more and in combination with progestin may modestly increase the risk for stroke [60, 61]. Recent clinical trials have demonstrated that menopausal hormone therapy for 5 to 7 years is not associated with risk of all-cause, cardiovascular, or cancer mortality among 27,347 postmenopausal women aged 50 to 79 years during a cumulative follow-up of 18 years [62]. In contrast to the decline in estrogen levels observed during menopausal transition, FSH levels rise sharply and may also contribute to increased risk of cardiovascular disease following the menopause (Figure 1). Several previous studies have examined associations between FSH levels and subclinical atherosclerosis including coronary artery calcium and the carotid intima-media thickness (IMT) in women, with the majority of them reporting a positive correlation [63-65]. However, in contrast with this observation, a recent study which included 2,658 Chinese postmenopausal women showed that serum FSH levels were negatively associated with 10-year risk of cardiovascular disease [66]. Such differences are likely related to distinct patient cohorts, baseline eligibility criteria, race, age and experimental

designs. Interestingly, a recent SWAN study evaluated the impact of the entire midlife course of hormonal trajectories on the risk of atherosclerosis after menopause, which suggested that women with a lower FSH rise over their menopause transition may be at lower risk of atherosclerosis than those with a medium or high FSH rises [67]. This study suggests that it may be much more important to examine the correlation between FSH trajectory and cardiovascular disease and osteoporosis, rather than ascribe risk to a single assessment of hormone at a single time point.

Potential mechanisms by which FSH increases risk of cardiovascular disease

FSH may increase risk of cardiovascular disease through a number of distinct mechanisms (Figure 4):

Effects of FSH on endothelial cell activation and atherosclerosis

Endothelial cell activation by oxidized lipids and pro-inflammatory stimuli plays an important role in the initiation and progression of atherosclerosis. Activated endothelial cells express a variety of adhesion molecules such ICAM-1 and VCAM-1, which recruits leukocytes to the endothelium [68]. Our recent report demonstrated for the first time that FSH directly accelerates atherosclerosis in ovariectomized *ApoE*^{-/-} mice, independent of estrogen deficiency. Mechanistically, FSH up-regulated VCAM-1 expression in human umbilical vein endothelial cells and subsequently increased human monocyte adhesion through FSHR/Gαs/cAMP/PKA and PI3K/Akt/mTOR/NF-κB signaling pathways. Interestingly, FSHR was specifically localized in **caveolae** of endothelial cells. Caveolin-1, the main protein component of caveolae, directly interacted with FSHR and mediated its downstream signaling actions [36].

FSH accelerates adiposity and lipid accumulation

Cui *et al* recently reported that FSH treatment increases the weight of abdominal fat and lipid accumulation of adipocytes in chickens, through enhancing the expression of genes in the fatty acid metabolism pathway, retinol metabolism pathway, and peroxisome proliferator-activated receptor (PPAR) signaling pathway [69]. Further studies showed that FSHR was functionally expressed in human and mouse fat tissues

and adipocytes. FSH treatment increased lipid biosynthesis, lipid droplet formation, altered the secretion of leptin and adiponectin through the $G\alpha i/Ca^{2+}/CREB$ pathway in mouse 3T3-L1 preadipocytes [70]. Interestingly, recent elegant studies performed by Liu *et al* have shown that blockade of FSH signaling with a specific antibody reduces high-fat diet-induced obesity in wild type mice and adiposity in ovariectomized mice [55]. Specifically, antibody-treated mice showed significantly reduced total (TFV), subcutaneous (SFV) and visceral fat volume, but interscapular brown adipose tissue remained unchanged [55]. These data suggest that FSH may play an important role in determining body fat distribution, which may contribute to cardiovascular dysfunction [71]. Interestingly, it has been shown that serum FSH levels are positively associated with serum total cholesterol and LDL-C levels in Chinese postmenopausal women [34]. FSH administration in ovariectomized mice promotes dyslipidemia through inhibiting hepatic cholesterol metabolism. Mechanistically, FSH reduces LDLR levels in hepatocytes and attenuates the endocytosis of LDL-C, leading to an elevated circulating LDL-C level [34], which is an independent risk factor for atherosclerosis. Taken together, these studies suggest that FSH may increase risk factors for cardiovascular disease through accelerating adiposity and lipid accumulation.

FSH promotes angiogenesis

Angiogenesis inhibition through the application of endostatin or TNP-470 has been shown to decrease plaque formation and intimal neovascularization in *ApoE*^{-/-} mice [72]. FSH signaling through FSHR in HUVECs promotes angiogenesis, which is independent of VEGF [73]. Interestingly, treatment of HUVECs with FSH stimulated the PI3K/AKT signaling pathway, with similar efficacy as VEGF [73]. These data suggest that FSH may increase atherosclerosis through promoting angiogenesis. However, the effect of FSH on angiogenesis cannot be reproduced by other investigators and the discrepancy remains to be clarified [37].

Potential role of FSH in vascular calcification.

Vascular calcification is a common feature of advanced atherosclerosis and is important

in determining atherosclerotic plaque stability [74]. We and others have previously demonstrated that vascular calcification shares many similarities to bone remodeling, which involves maintaining a balance between osteoblasts and osteoclasts [75-77]. The presence of osteoclast-like cells and active resorption of ectopic mineral deposition have been reported in calcified atherosclerotic plaques [78]. Furthermore, monocytes and macrophages are frequently observed in atherosclerotic plaque [79]. These data suggest that osteoclasts, through a signaling cascade involving FSH (Figure 3) may be recruited to calcified regions where they can resorb mineral deposition, subsequently increasing the incidence of plaque rupture [80] (Figure 4). In addition, FSH stimulates immune cells to release inflammatory cytokines [53, 54], which may also contribute to vascular calcification (Figure 4).

Clinical use and long-term safety of recombinant FSH therapies against cardiovascular disease and osteoporosis

There are currently three commercially available recombinant human FSH therapies, namely Gonal-F, Puregon and Elonva. They are normally prescribed for women with subfertility who require assisted reproductive technology. Whether treatment with these medications has long-term safety issues is still under considerable debate. A previous study showed that there is no association between successful fertility treatment using FSH and increased risk of cardiovascular disease later in life [81]. However, several recent studies have suggested that following fertility therapy women may have an increased risk of long term adverse cardiovascular events, such as hypertension and stroke [82, 83]. To our knowledge, there are no data about fertility treatment using recombinant FSH therapies and the long-term risk of osteoporosis in women to date, and requires future interrogation in the future. Given the current high demand for fertility treatment, a greater understanding of the long-term risks of fertility treatment is essential for the development of clinical strategies targeting osteoporosis and cardiovascular risk reduction.

Concluding remarks and future perspectives

In the current review, we have highlighted our current understanding of the potential pathological roles for FSH in women. Epidemiological and animal studies have revealed that FSH contributes to the decline in bone health in women undergoing menopausal transition. Emerging data has shown that FSH treatment enhances atherosclerotic plaque formation in ovariectomized AopE^{-/-} mice [36]. However, our understanding of the molecular mechanisms through which FSH increases the risk of osteoporosis and cardiovascular disease is far from complete (see outstanding questions). The vast technological advancements such as Next-Generation Sequencing will provide valuable new information on the molecular pathophysiology of FSH in extragonadal tissues, such as bone and blood vessels. Animal studies have shown that blocking FSH increases bone mass, reduces body fat and induces thermogenic adipose tissue. These data suggest that blocking FSH may not only be a potential opportunity for treating osteoporosis, but also of benefit in the treatment of visceral fat associated diseases such as metabolic syndrome and cardiovascular disease [55]. Whether FSH promotes osteoporosis and cardiovascular disease in women remains to be further elucidated in humans. In conclusion, a fuller understanding of the molecular pathological roles of FSH for osteoporosis and cardiovascular disease in postmenopausal women could have significant importance in improving diagnosis, management and treatment of these major diseases.

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542 **Additional materials**

Box1. The regulation of FSH production and its activity

FSH is composed of a common α subunit and a hormone-specific β subunit, which are non-covalently associated. It is only biologically active when these two subunits are fused together [16]. FSH levels are strictly controlled by the hypothalamic pituitary gonadal axis and sex hormone-mediated negative feedback. In response to gonadotropin-releasing hormone from the hypothalamus, pituitary gonadotrophs produce FSH and LH [17], which exert their primary function in the gonads. In the ovary, FSH stimulates the expression of aromatase, which converts androgens to estrogens and induces follicular maturation. In the testes, it acts together with testosterone to support spermatogenesis [18]. The estrogens in females and androgens in males feedback to the hypothalamus and pituitary, and suppress the production of FSH [19]. In addition, previous studies have shown that inhibin A and B also inhibits the production of FSH in a manner of negative feedback [20, 21]. Similar to other glycoprotein hormones, FSH has various **glycosylation** variants with differential oligosaccharide composition, which contribute nearly 30% of the FSH mass and are located in both the α and β subunits [22]. Differential glycosylation of FSH modulates its function, including folding, intracellular trafficking, secretion, receptor binding, heterodimer stability and cell signaling [15].

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Box2. FSH intracellular signaling pathways

It is now well recognized that upon FSH binding, the activated FSHR triggers a number of intracellular signaling pathways either in parallel or sequentially. Classically, upon FSH activation, FSHR functionally couples to G α s subunit and induces the cAMP/PKA signaling pathway, subsequently activating the cAMP regulatory element-binding protein (CREB) and modulating gene expression, such as aromatase and inhibin- α [24]. This canonical signaling pathway plays a key role for its biological actions inside granulosa cells in the ovary and sertoli cells in the testes. It is now clear that FSHR also involves other transduction mechanisms upon FSH binding. In recent years, a number of G α s-independent transduction mechanisms have been found. It has been reported that FSHR also couples to G α i [25], G α q [26], β -arrestins [27], Adapter protein containing the Pleckstrin homology domain, the Phosphotyrosine binding domain, and the Leucine zipper motif (APPL) [28-30]. These FSHR interacting partners enable FSH to activate alternative signaling pathways, including those mediated by distinct kinases such as inositol trisphosphate (IP3), Akt and ERK1/2. This complex signaling network triggered by FSH fine-tunes its biological action and determines the fate of target cells (Figure 2).

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Glossary

Amenorrhea: the absence of a menstrual period in a woman of reproductive age.

Bone resorption: the process that osteoclasts resorb bone tissue and release the minerals.

Caveolae: the small invaginations of the plasma membrane of cells, belonging to a special type of lipid raft.

Menopausal transition (STRAW +10 Staging System: Stages -2 to -1) [ref. 1]

Early menopausal transition (Stage -2): Criterias include increased variability in menstrual cycle length, a persistent difference of 7 days or more in the length of consecutive cycles, elevated but variable FSH levels, low antimüllerian hormone and antral follicle count.

Late menopausal transition (Stage -1): Criterias include the occurrence of amenorrhea of 60 days or longer, elevated FSH levels greater than 25 mIU/mL, low antimüllerian hormone and antral follicle count, and vasomotor symptoms (likely).

Perimenopause (STRAW +10 Staging System: stage -2 to +1a): the time around menopause and begins at Stage -2 and ends 12 months after the final menstrual period (Stage +1a).

Postmenopause (STRAW +10 Staging System: Stage +1a, +1b, +1c, +2): The time after the final menstrual period.

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Figure legends

Figure 1. Ovarian hormonal changes in perimenopause. With transition into menopause, FSH increases markedly and estrogen declines. In postmenopausal women, estrogen levels are low, and FSH levels remain high for several years [2]. This graph is intended to show the trends of changes in FSH and Estrogen levels and do not stand for absolute amounts.

Figure 2. Multiple intracellular signaling pathways induced by FSH. Classically, upon FSH binding, the activated FSHR induces canonical Gas/cAMP/PKA signaling pathway, which subsequently activates CREB and regulates target gene expression. FSHR also activates its downstream signaling pathways through other Gas-independent transduction mechanisms mainly coupling to other G α subunits, β -arrestin-dependent signaling, EGFR transactivation, and APPL1-mediated signals [24-30].

Figure 3. FSH accelerates bone resorption through increasing osteoclast formation. FSH directly enhances RANK expression in monocytes [50-52]. RANKL released by osteoblasts or T cells binds to RANK in monocytes and promotes osteoclasts formation. FSH also stimulates monocytes and macrophages to release inflammatory cytokines such as TNF α , IL1 β and IL6 [53-54]. These inflammatory cytokines stimulate osteoblasts and T cells to secrete RANKL, which promotes osteoclasts formation. Osteoclasts are the cells responsible for bone resorption, and osteoblasts are the cells for bone formation. Increased ration of osteclasts to osteoblasts leads to bone mineral loss, which may subsequently cause osteoporosis [49].

Figure 4. The potential mechanisms through which FSH accelerates atherosclerosis. FSHR is expressed by endothelial cells. FSH induces the expression of vascular cell adhesion molecule 1 (VCAM-1), which recruits monocytes from circulation [36]. The recruited monocytes migrate and differentiate into macrophages. Lipid accumulates within these macrophages, leading to foam cell formation. Macrophage-derived foam cells contribute to the initiation, development and

587 progression of atherosclerosis. In addition, the recruited monocytes may also
588 differentiate into osteoclasts, which can resorb the calcified region and contribute to
589 plaque instability. Moreover, FSH may stimulate monocytes and T cells to release
590 inflammatory cytokines such as $\text{TNF}\alpha$, $\text{IL1}\beta$, IL6 [53-54], leading to chronic
591 inflammation, which may promote atherosclerosis and vascular calcification.

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Outstanding questions

Do other bone or vascular cells express FSHR, such as osteoblasts, osteocytes, vascular smooth muscle cells, adventitial cells and cardiomyocytes? What are the effects of FSH on these cells?

Are there any other downstream genes or signaling pathways activated by FSH in bone cells or cardiovascular cells responsible for its effects on osteoporosis and cardiovascular disease?

Do osteoclast or vascular endothelial cell specific FSHR conditional knockout mice demonstrate improved bone and vascular health?

Are there any other mechanisms involved in FSH enhanced atherosclerosis in ovariectomized ApoE^{-/-} mice such as induction of inflammation or foam cell formation?

FSH blocking increases bone mass and reduces body fat in mouse models. Can these findings be translated into humans?

Are there any potential mechanisms for FSH biological activities without binding to FSHR in extragonadal tissues?

What are the effects of glycosylation state of FSH on its actions in extragonadal tissues?

Figure 1

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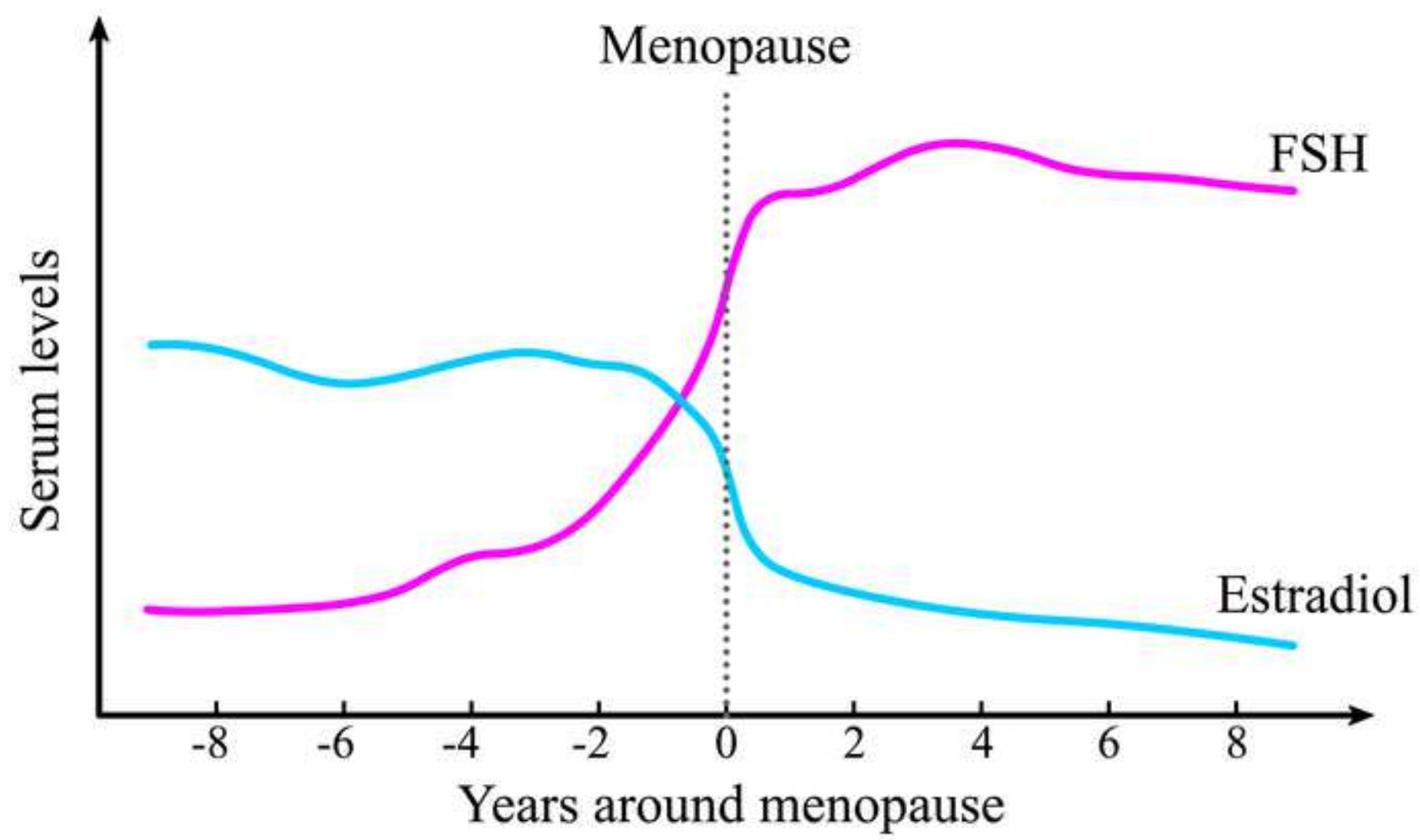
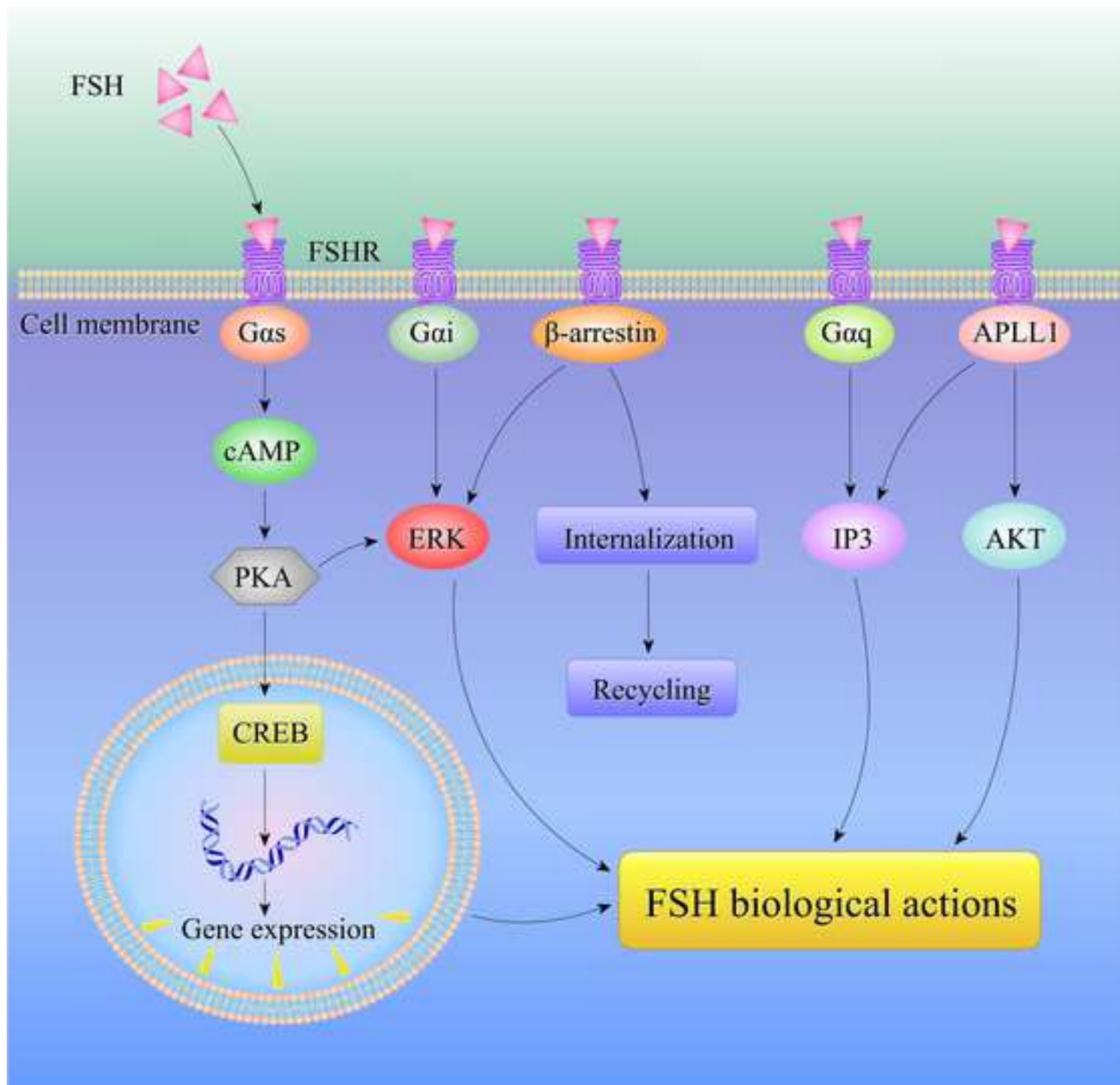


Figure 2

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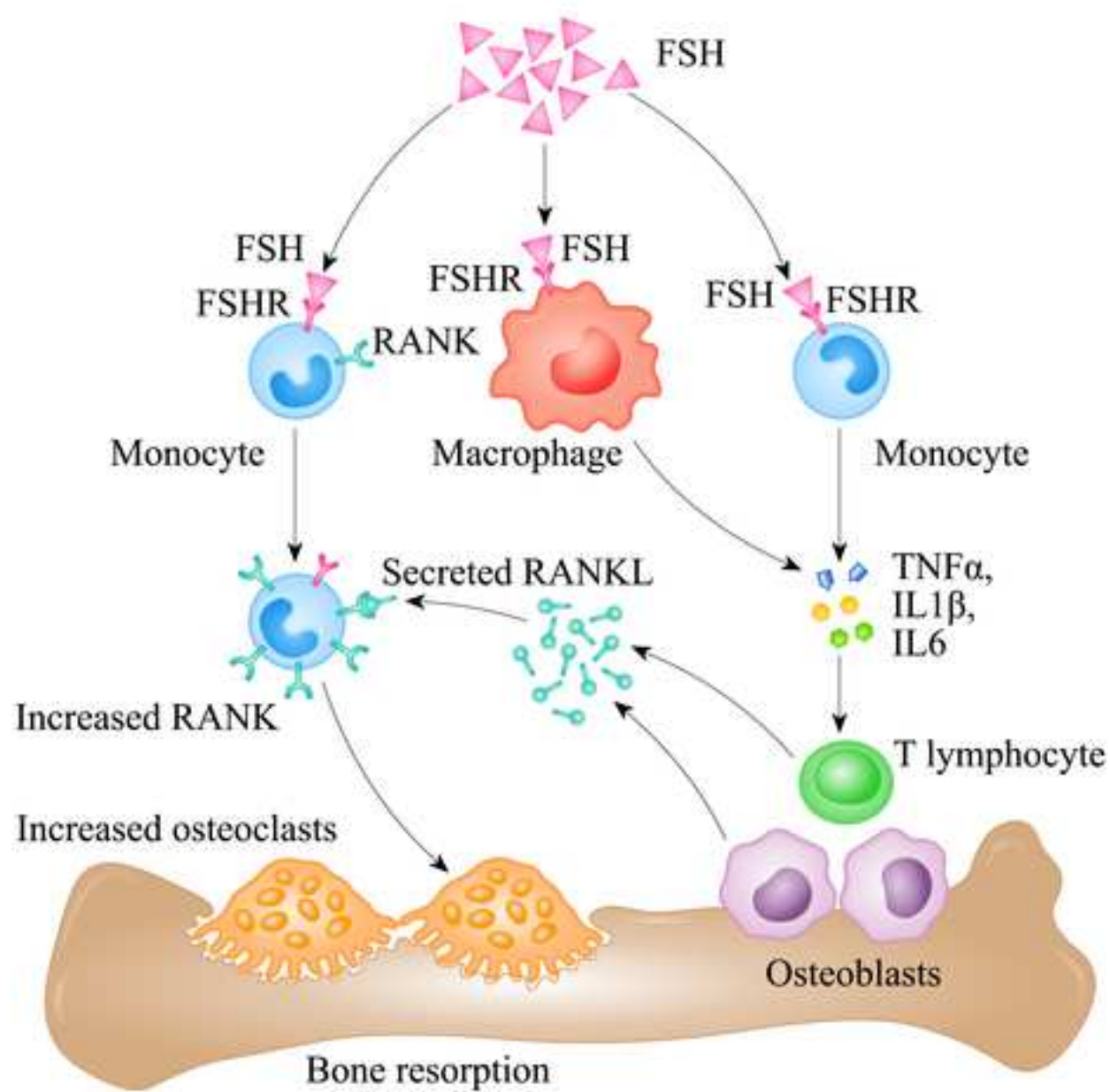
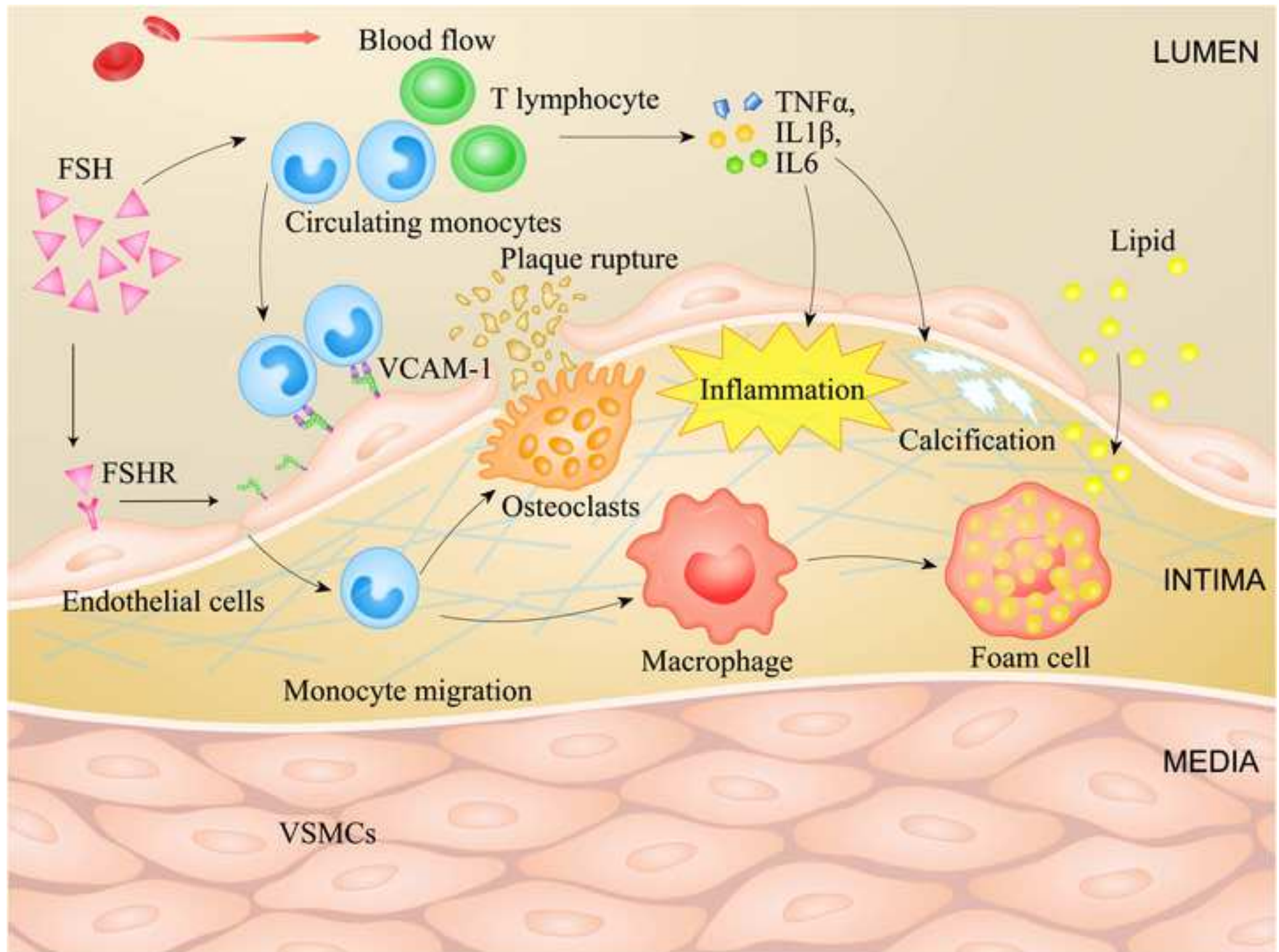


Figure 4



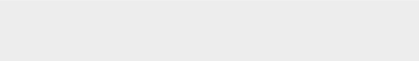
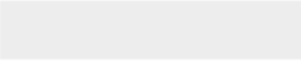






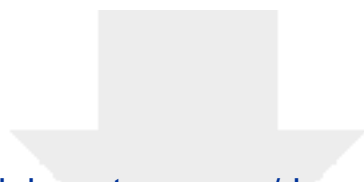
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